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(57) Abstract

A method and means for providing functional equivalents to organs wherein cells are grown on polymer scaffolding using cell culture techniques followed by transfer of the polymer-cell scaffold into a patient at a site appropriate for attachment, growth and function, after growth and vascularization. Once the structure is implanted and vascularization takes place, the resulting organ is a blend of the parenchymal elements of the donated tissue and vascular and matrix elements of the host. A key element of the method is the design and construction of the polymer scaffold using a material and shape that provides for attachment and growth of the cells such that an adequate exchange of nutrients, wastes and gases occurs by diffusion even within the inner layers of the cells, until such time as implantation and vascularization occur.

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transplantation of islet cells through injection of isolated clusters of islet cells into the portal circulation, with implantation in the vascular bed of the liver. More recent experimental methods have included encapsulation of pancreatic beta cells to prevent immune attack by the host and injection of fetal beta cells beneath the capsule of the kidney. Although there is evidence of short term function, long term results have been less satisfactory (D.E.R. Sutherland, Diabetologia 20, 161-185 (1981); D.E.R. Sutherland, Diabetologia 20,435-500 (1981)). Currently whole organ pancreatic transplantation is the preferred treatment.

There are also many diseases which cause significant scarring of the liver, ultimately causing hepatic failure. There are no artificial support systems for liver failure, so that, in the absence of a successful transplant, liver failure always results in the death of the patient. It has been estimated that 30,000 people die of hepatic failure every year in the United States, at a cost to society of $14 billion dollars annually.

There are many diseases which are termed "inborn errors of metabolism", including genetic defects that result in defects of protein metabolism, defects of amino acid metabolism, defects of carbohydrate metabolism, defects of pyrimidine and purine metabolism, defects of lipid metabolism, and defects of mineral metabolism. A large number of these diseases are based in defects within the liver itself. Many of these patients have a structurally normal liver or reasonably normal liver at the time diagnosis is made. Many of the diseases, in fact, do not damage

large. For example, in the Baltimore area during 1973 the age adjusted incidence rates for all alcoholic liver diseases per 100,000 population over 20 years were: 36.3 for white males, 19.8 for white females, 60.0 for nonwhite males, and 25.4 for nonwhite females. The morbidity for liver cirrhosis has been reported to be twenty-eight times higher among serious problem drinkers than amongst nondrinkers in a survey of factory workers. There is a direct correlation between the amount of alcohol consumed and the incidence of cirrhosis. The mortality rates for cirrhosis vary greatly from country to country, ranging from 7.5 per 100,000 in Finland to 57.2 per 100,000 in France. In the U.S., the trend has been alarming in terms of increasing incidence of alcoholic cirrhosis and death. Between 1950 to 1974, deaths from cirrhosis in the U.S. increased by 71.7% while deaths from cardiovascular diseases decreased by 2%. At this time, these patients have no options.

There are many other vital organ systems for which there is no adequate means for replacement or restoration of lost function. For example, in the past, loss of the majority of intestine was a fatal condition. Although patients can now be supported totally with nutrition supplied via the veins, this is thought of as a "half-way technology" because of the many complications associated with this technique. One problem is that, over time, many patients on total parenteral nutrition develop irreversible liver disease and die of their liver disease. Other patients develop severe blood stream infections requiring multiple removal and replacement procedures. They may eventually lose all available veins and succumb of malnutrition or die of infection.

Selective cell transplantation of only those parenchymal elements necessary to replace lost function has been proposed as an alternative to whole or partial organ transplantation (P.S.Russell, Ann. Surg. 201(3),255-262 (1985)). This has several attractive features, including avoiding major surgery with its attendant blood loss, anesthetic difficulties, and complications. It replaces only those cells which supply the needed function and, therefore, problems with passenger leukocytes, antigen presenting cells, and other cell types which may promote the rejection process are avoided. Adding the techniques of cell culture provides another set of tools to aid in the transplantation process. The ability to expand cell numbers with proliferation of cells in culture, in theory, allows autotransplantation of one's own tissue. For example, hepatocyte injections into the portal circulation have been attempted to support hepatic function. A recent novel approach in which hepatocytes were attached to collagen coated microcarrier beads prior to injection into the peritoneal cavity demonstrated successful implantation, viability of the implanted hepatocytes, and function, as described by A.A.Demetriou,et al., Science 233,1190-1192 (1986).

Loss of other types of organ or tissue function such as muscle or nervous tissue can also lead to deforming illnesses and social tragedies. Methods of muscle and nerve transfer have been developed by surgeons through the last fifty years which are ingenious in design. An example of a technique for restoring nerve function has been to string dead nerve fibers from nerve centers to places with lost nerve function. Many other disorders of the nervous system

skin-equivalent. U.S. Patent No. 4,060,081, tc Yannas et al. discloses a multilayer membrane useful as synthetic skin which is formed from an insoluble non-immunogenic material which is nondegradable in the presence of body fluids and enzymes, such as cross-linked composites of collagen and a mucopolysaccharide, overlaid with a non-toxic material such as a synthetic polymer for controlling the moisture flux of the overall membrane. U.S. Patent No. 4,458,678 to Yannas et al. discloses a process for making a skin-equivalent material wherein a fibrous lattice formed from collagen cross-linked with glycosaminoglycan is seeded with epidermal cells.

A disadvantage to the first two methods is that the matrix is formed of a "permanent" synthetic polymer. The '678 patent has a feature that neither of the two prior patents has, a biodegradable matrix which can be formed of any shape, using the appropriate cells to produce an organ such as the skin. Unfortunately, there is a lack of control over the composition and configuration of the latter matrices since they are primarily based on collagen. Further, since collagen is degraded by enzymatic action as well as over time by hydrolysis, the degradation is quite variable. Moreover, the matrix is completely infiltrated with cells and functional in the absence of the moisture controlling polymer overlay only when it is grafted onto the patient and capillaries have formed a vascular network through the entire thickness of the matrix. The limitation of these matrices as a function of diffusion is discussed in the article by Yannas and Burke in J.Biomed.Mater.Res., 14, 65-81 (1980) at page 73. Although the authors recognized that the pore size and

blood vessels, nerves, and muscles, which functionally resemble the naturally occurring organ.

It is a further object of the present invention to provide a method and means for designing, constructing and utilizing artificial matrices as temporary scaffolding for cellular growth and implantation.

It is a still further object of the invention to provide biodegradable, non-toxic matrices which can be utilized for cell growth, both *in vitro* and *in vivo*, as support structures in transplant organs immediately following implantation.

It is another object of the present invention to provide a method for configuring and constructing biodegradable artificial matrices such that they not only provide a support for cell growth but allow and enhance vascularization and differentiation of the growing cell mass following implantation.

It is yet another object of the invention to provide matrices in different configurations so that cell behavior and interaction with other cells, cell substrates, and molecular signals can be studied *in vitro*.

Summary of the Invention

The present invention is a method and means whereby cells having a desired function are grown on polymer scaffolding using cell culture techniques, followed by transfer of the polymer-cell scaffold into a patient at a site appropriate for attachment, growth and function, after attachment and equilibration, to produce a functional organ equivalent. Success depends on the ability of the implanted cells to

and gases, so as to produce uniform cell growth and proliferation. Theoretical calculations of the maximum cell attachment suggest that fibers 30 microns in diameter and one centimeter in length can support 125,000,000 cells and still provide access of nutrients to all of the cells. Another advantage of the biodegradable material is that compounds may be incorporated into the matrix for slow release during degradation of the matrix. For example, nutrients, growth factors, inducers of differentiation or de-differentiation, products of secretion, immunomodulators, inhibitors of inflammation, regression factors, biologically active compounds which enhance or allow ingrowth of the lymphatic network or nerve fibers, and drugs can be incorporated into the matrix or provided in conjunction with the matrix, in solution or incorporated into a second biodegradable polymer matrix.

Cells of one or more types can be selected and grown on the matrix. The matrix structure and the length of time and conditions under which the cells are cultured <u>in vitro</u> are determined on an individual basis for each type of cell by measuring cell attachment (only viable cells remain attached to the polymers), extent of proliferation, and percent successful engraftment. Examples of cells which are suitable for implantation include hepatocytes and bile duct cells, islet cells of the pancreas, parathyroid cells, thyroid cells, cells of the adrenal-hypothalmic-pituitary axis including hormone-producing gonadal cells, epithelial cells, nerve cells, heart muscle cells, blood vessel cells, lymphatic vessel cells, kidney cells, and intestinal cells, cells

Brief Description of the Drawings

Figure 1 is a schematic of the process of the present invention to produce a chimeric organ, in this diagram, a liver, pancreas or intestine: (1) the appropriate parenchymal cells are harvested, dispersed, and seeded onto the polymer matrix in cell culture, where attachment and growth occur and (2) a partial hepatectomy is performed to stimulate growth of the transplant and the polymer-cell scaffold is then implanted into the recipient animal where neovascularization, cell growth, and reabsorption of the polymer matrix occurs.

Figure 2 are the chemical structures of polymers which have been used for biodegradable cellular matrices: (a) polygalactin; (b) polyorthoester; and (c) polyanhydride.

Figure 3 is a diagram demonstrating the slow release of biologically active factors from the polymer matrix.

Figure 4 is a diagram of a technique to study in vitro morphogenesis using biodegradable polymers, cells, and matrix.

Figure 5 is a photograph (172x) of hepatocytes attached to fibers of polyglactin 910 after 4 days in culture. Cells are stained with Hematoxylin and Eosin.

Figure 6 is a photograph of bile duct epithelial cells cultured on polymer fibers for one month.

Figure 7 is a photograph (172X) of an implant of hepatocytes from an adult rat donor into omentum. The polymer-cell implant has been in place for 7 days before sacrifice. Hepatocytes are healthy and several mitotic figures can be seen. Blood vessels are

Figure 14 is a photograph of bovine aortic endothelial cells attached to polymer fibers after one month in culture.

Figure 15 is a phase contrast photomicrogarph showing polymer fibers coated with mouse fetal fibroblasts. The fibroblasts can be seen streaming off the polymer fibers in a straight line onto the culture dish.

Figure 16 is a phase contrast photomicrograph of polymer fibers coated with mouse fetal fibroblasts. These fetal fibroblasts have migrated off of the polymer through media and have attached at the bottom of the tissue culture plate.

Figure 17 is a scanning electron micrograph (472x) of a polyanhydride fiber immersed in a phosphate buffer solution, indicating that immersion of polymer fibers in differing buffers can alter the polymer surface and, therefore, influence cell attachment and differentiation.

Figure 18 is a scanning electron micrograph (493x) of polymer fibers coated with 1% gelatin, showing that the polymer fibers can be coated with cell adhesion agents to increase cell attachment.

Figure 19 is a perspective drawing of a bioabsorbable polymer fiber used for growth of nerve cells.

Figure 20a is a plan drawing of polymer spicules seeded with heart muscle cells and implanted on the myocardium of the heart.

Figure 20b is an enlarged plan view of a spicule, as shown in Figure 20a.

Figure 21a is a cross sectional view of wells containing various thicknesses of collagen (0, 3.0 mm,

are placed and the degree of alteration which they have undergone.

3. Tissue cannot be implanted in volumes greater than approximately one to three mm^3, because nutrition is supplied by diffusion until new blood vessels form, and this distance is the maximum distance over which diffusion can transpire until angiogenesis occurs.

4. Cell shape is determined by cytoskeletal components and attachment to matrix plays an important role in cell division and differentiated function. If dissociated cells are placed into mature tissue as a suspension without cell attachment, they may have a difficult time finding attachment sites, achieving polarity, and functioning because they begin without intrinsic organization. This limits the total number of implanted cells which can remain viable to organize, proliferate, and function.

The latter principle is a key point in the configuration of the support matrices. For an organ to be constructed in tissue culture and subsequently successfully implanted, the matrices must have sufficient surface area and exposure to nutrients such that cellular growth and differentiation can occur prior to the ingrowth of blood vessels following implantation. After implantation, the configuration must allow for diffusion of nutrients and waste products and for continued blood vessel ingrowth as cell proliferation occurs.

This method for replacing or supplementing lost organ function has a number of advantages over either pharmacologic manipulation or transplantation of whole organs or parts of organs. Although great strides have been made in these areas, the results of these

remaining 10% can be harvested and cultured. The cells expand in a logarithmic fashion in culture. The cells are cultured until suitable numbers of cells are achieved, the cells are grown onto the appropriate polymer scaffold, and placed back into the patient, to be allowed to vascularize, grow and function as a neointestine.

In the case of liver function replacement, it may be possible to construct a cell-matrix structure without the absolute need for hepatocyte proliferation in culture. This hypothesis is based on the observation that a high yield of hepatocytes can be obtained from a small piece of liver. For example, in experiments on 250 gm rats, it is known that the liver weighs approximately 12 gm. At a 90% viability rate this yields 2.5×10^8 viable hepatocytes. It is also thought that only 10% of hepatic cell mass is necessary for cell function. Therefore, for a 250 gm rat, 1.2 gm of tissue is needed, an implant of approximately 2.5×10^7 cells. This assumes no proliferation in vivo. Implants into children as well as adults are theoretically possible. An 8 month child has a normal liver that weighs approximately 250 gm. That child would, therefore, need 25 gm of tissue from a biopsy from a parent. An adult liver weighs approximately 1500 gm, therefore, the biopsy would only be about 1.5% of his liver or 5.0×10^8 cells. Again, this assumes no proliferation. An adult would need a larger biopsy which would yield about 2.5×10^9 cells. If these cells are attached with high efficiency and implanted, proliferation in the new host should occur. The resulting hepatic cell mass should be adequate to replace needed function.

In distinct contrast to the prior art, the present method uses a temporary scaffolding for controlled growth and proliferation of cells in vitro, followed by implantation of functional cells into patients. The result is an organ which is vascularized in vivo to allow growth of the cells in a three-dimensional configuration similar to that of the organ whose function they are replacing. Both the design and construction of the scaffolding, as well as the conditions of the initial cell culture, are used to encourage cells to achieve their biological potential and replicate the ontogeny of organ formation which occurs in embryonic and fetal life. As described herein, this technique is termed chimeric neomorphogenesis.

The design and construction of the scaffolding is of primary importance. The matrix should be shaped to maximize surface area to allow adequate diffusion of nutrients and growth factors to the cells. The maximum distance over which adequate diffusion through densely packed cells can occur appears to be in the range of approximately 100 to 300 microns under conditions similar to those which occur in the body, wherein nutrients and oxygen diffuse from blood vessels into the surrounding tissue. The actual distance for each cell type and polymer structure must be determined empirically, measuring cell viability and function in vitro and in vivo. This determination for bovine capillary endothelial cells in combination with a collagen matrix will be described in detail in a subsequent example.

The cells are initially cultured using techniques known to those skilled in the art of tissue culture. Once the cells have begun to grow and cover

normally be achieved where nutrients are supplied solely by diffusion.

All polymers for use in the present invention must meet the mechanical and biochemical parameters necessary to provide adequate support for the cells with subsequent growth and proliferation. The polymers can be characterized with respect to mechanical properties such as tensile strength using an Instron tester, for polymer molecular weight by gel permeation chromatography (GPC), glass transition temperature by differential scanning calorimetry (DSC) and bond structure by infrared (IR) spectroscopy; with respect to toxicology by initial screening tests involving Ames assays and *in vitro* teratogenicity assays, and implantation studies in animals for immunogenicity, inflammation, release and degradation studies.

In vitro cell attachment and viability can be assessed using scanning electron microscopy, histology, and quantitative assessment with radioisotopes.

The configuration of the polymer scaffold must have enough surface area for the cells to be nourished by diffusion until new blood vessels interdigitate with the implanted parenchymal elements to continue to support their growth, organization, and function. Polymer discs seeded with a monolayer of cells, and branching fiber networks both satisfy these needs.

At the present time, a fibrillar structure is preferred. The fibers may be round, scalloped, flattened, star shaped, solitary or entwined with other fibers. The use of branching fibers is based upon the same principles which nature has used to solve the problem of increasing surface area

compounds which enhance or allow ingrowth of the lymphatic network or nerve fibers, and drugs, can be incorporated into the matrix or provided in conjunction with the matrix, as diagrammed in Figure 3.

The branching fibers 14 shown in Fig. 1, when 30 microns in diameter and 1.0 cm in length, can theoretically support 125,000,000 cells. In the example in which a liver organ is constructed, the cell populations can include hepatocytes and bile duct cells. Cells may be derived from the host, a related donor or from established cell lines. Fetal cells lines may be utilized since these cells are generally more hardy than other cell lines.

In one variation of the method using a single matrix for attachment of one or more cell lines, the scaffolding is constructed such that initial cell attachment and growth occur separately within the matrix for each population. Alternatively, a unitary scaffolding may be formed of different materials to optimize attachment of various types of cells at specific locations. Attachment is a function of both the type of cell and matrix composition.

Although the presently preferred embodiment is to utilize a single cell-matrix structure implanted into a host, there are situations where it may be desirable to use more than one cell-matrix structure, each implanted at the most optimum time for growth of the attached cells to form a functioning three-dimensional organ structure from the different cell-matrix structures. In some situations, it may be desirable to prepare the implantation site by initially exposing the cells at the site to a biodegradable polymer matrix including compounds or

the systemic circulation through the hemiazygous system. If this occurs, there would be portosystemic channels through the implanted hepatic cells which may allow for decompression of portal hypertension, a complication leading to gastrointestinal bleeding in patients with end-stage liver disease.

In the case of metabolic liver disease, where the native liver is structurally normal and can drain bile, appropriate hepatocytes on scaffolds can be placed directly into the recipient liver. This intrahepatic engraftment would occur in relation to the normal host biliary system. The native liver would then be a chimera of patient cells and donor cells draining into the patient's biliary tree.

For this procedure to be successful, the function of the implanted cells, both in vitro as well as in vivo, must be determined. In vivo liver function studies can be performed by placing a cannula into the recipient's common bile duct. Bile can then be collected in increments. Bile pigments can be analyzed by high pressure liquid chromatography looking for underivatized tetrapyrroles or by thin layer chromatography after being converted to azodipyrroles by reaction with diazotized azodipyrroles ethylanthranilate either with or without treatment with β-glucuronidase. Diconjugated and monoconjugated bilirubin can also be determined by thin layer chromatography after alkalinemethanolysis of conjugated bile pigments. In general, as greater numbers of functioning transplanted hepatocytes implant, the levels of conjugated bilirubin will increase. The same technique measuring monoconjugated and diconjugated bilirubin can be performed in vitro by testing the media for levels of these bilirubin

with hepatocytes; 23 with intestinal cells and clusters; and 26 with pancreatic islet preparations. The cells remained viable in culture, and in the case of fetal intestine and fetal hepatocytes, appeared to proliferate while on the polymer. After 4 days in culture, the cell-polymer scaffolds were implanted into host animals, either in the omentum, the interscapular fat pad, or the mesentery. In 3 cases of fetal intestinal implantation coupled with partial hepatectomy, successful engraftment occurred in the omentum, one forming a visible 6.0 mm cyst. Three cases of hepatocyte implantation, one using adult cells and two using fetal cells, have also engrafted, showing viability of hepatocytes, mitotic figures, and vascularization of the cell mass.

Materials and Methods

Polymers:

Three synthetic absorbable polymers were used to fabricate filaments and discs as matrices for cell attachment, growth, and implantation (Fig. 2).

1. Polyglactin. This polymer, developed as absorbable synthetic suture material, a 90:10 copolymer of glycolide and lactide, is manufactured as Vicryl® braided absorbable suture (Ethicon Co., Somerville, New Jersey) (Craig P.H., Williams J.A., Davis K.W., et al.: A Biological Comparison of Polyglactin 910 and Polyglycolic Acid Synthetic Absorbable Sutures. Surg. 141; 1010, (1975)).

2. Polyorthoesters. The specific polymer used was: 3,9-bis(ethylidene-2, 4, 8, 10-tetraoxaspiro[5.5] undecane copolymer with tran-1,4-cyclohexanedimethanol and 1,6-hexandiol in a molar ratio 2:1:1, respectively (SRI, California) (Heller J., Penhale W.H., Helwing

branching pattern relief, 10 mm in diameter and 0.5 mm thick.

C. Filament Drawing. Filaments were drawn from the molten polymer (30 microns in diameter). Small flattened 1.0 cm. tufts were used for the experiments.

D. Polyglactin 910. Multiple fibers of 90:10 copolymer of glycolide and lactide converging to a common base were fashioned from suture material of Q-Vicryl® by fraying the braided end of the polymer. These branching fiber clusters were approximately 1.0 cm. in height. The individual fibrils were 30 microns in diameter.

Animals:

Young adult and fetal Sprague-Dawley rats and C57 Bl/6 mice (Charles River Labs, Wilmington, Massachusetts) were used as cell donors for all experiments. The animals were housed individually, allowed access to food and water ad lib, and maintained at 12 hour light and dark intervals. Animals were anesthetised with an IP injection of pentobarbital (Abbott Labs, North Chicago, Illinois) at a dose of 0.05 mg/g and supplemented with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, New Jersey) by cone administration. Fetal animals were harvested at 13, 17 and 20 days gestation for use as liver, pancreas, and intestinal donors. Young adult animals were used as liver and pancreas donors and as recipients of the cell-scaffold matrices.

Cell Harvest and Cell Culture

Liver:

After the induction of anesthesia, the abdomen of young adult animals was shaved, prepped with betadine, and opened using sterile technique. The

animals of the appropriate gestation. The intact uterus with multiple fetuses was transferred in saline to a sterile room, equipped with a dissection microscope. Individual fetuses were opened and the liver, intestine, and pancreas were harvested and pooled. Organs were then transferred to a sterile hood for cell isolation. The tissues were minced, treated with a 0.025% Type II collagenase, and dispersed into cell suspensions.

Pancreas:

After the induction of anesthesia, the abdomen of young adult animals was shaved, prepped with betadine, and opened in the midline using sterile technique. The common bile duct was isolated, and the pancreas visualized. 2.5 cc.'s of 2.0% Type II collagenase (BCA/Cappel Products, West Chester, Pennsylvania), was infused into the pancreas by injection into the common bile duct using the technique described by Gotoh et al. (Gotoh M., Maki T., Kiyozumi T., et al.: An Improved Method of Isolation of Mouse Pancreatic Islets. Trans. 40; 4, pp. 436-438, 1985). After 5 minutes, the pancreas was transferred to a sterile hood for islet cell isolation. Briefly, the tissue was placed into a 25% Ficoll solution and layered under a discontinuous Ficoll gradient (23, 21, 11%) and centrifuged at 800 x g. for 10 minutes. Islets which aggregated at the 21-11% interface were washed with cold Hank's solution and centrifuged at 320 x g. 3 times. The islets were resuspended in RPMI 1640 (Gibco, Grand Island, New York) media-supplemented with 10% fetal calf serum, and overlaid onto polymer scaffolds. Fetal animals were harvested as donors as described above.

fluorescine isothyocyanate (FITC) antisera labelled to the appropriate monoclonal antibody, for example, the HY antigen or other markers of hepatocyte membranes, are applied individually to separate moist biopsy sections. They are incubated at room temperature for 30 minutes in a moist chamber. Following rinses with PBS the sections are cover-slipped with a glyceral-PBS mixture and examined using an immunofluorescence microscope (Leitz) with epi-illumination and a high pressure mercury lamp as the light source.

Electron microscopy: samples for electron microscopy are obtained from fresh tissue and fixed in 2% glutaraldehyde, post-fixed in 1% osmiumtetroxide, dehydrated in graded alcohols, and imbedded in epon-8:12. One micron thick section of the plastic imbedded tissue are made from areas of interest. Selected blocks are trimmed, ultrathin sections made, and stained with uranyl acetate and lead citrate, and examined with a Phillips 300X electron microscope.

Scanning electron microscopy (SEM): After hepatocytes are isolated and attached to the appropriate polymer, they are incubated for the appropriate interval. After culture, samples are prepared for SEM by incubating in a 50:50 solution of 2% glutaraldehyde phosphate buffer solution for 1 hour, the samples are then rinsed 4 times in PBS for 10 minutes per rinse to remove excess glutaraldehyde solution. Samples are dehydrated using progressively increasing ethanol solutions. Samples are then placed in a critical point dryer where ethanol is exchanged for liquid CO_2. Temperature is gradually increased to the critical point, ensuring dehydration. The samples are then coated with a thin layer of gold and placed under high vacuum in the scanning electron microscope.

phase and a chronic foreign body reaction to the implanted polymers. The intensity of inflammation varied with the polymer type tested: polyanhydride elicited the most severe acute and chronic response although the inflammation surrounding branching fibers of either polyorthoester or polyglactin appeared proportionately greater than the disc configuration because of the greater surface area of exposed foreign material to host.

Histologic examination of liver cell implants in 3 animals showed evidence of successful engraftment of hepatocytes at seven days, as shown by Figure 7. Small clusters of healthy appearing hepatocytes were seen with bile canaliculi between adjacent cell membranes and some areas demonstrated mitotic figures. The cells were surrounded by an inflammatory response and blood vessels coursed around and through the cell clusters. Polymer material was seen immediately adjacent to the cells.

Successful engraftment of intestinal cells and clusters were observed in 3 animals. Histologic findings were similar to the hepatocyte implants. On gross examination of the implant at 7 days, a cystic structure approximately 6.0 mm in length was found at the implant site with polymer fibers displayed within its wall (Figure 10). Microscopic examination revealed well differentiated intestinal epithelium lining the cavity with mucous and cellular debris within the lumen, shown in Figure 11. One wall of the cyst contained polymer fibers, blood vessels, and inflammatory cells immediately adjacent to the intestinal epithelium. The other wall included a muscular coating which suggested that the polymer held a small minced piece of fetal intestine as the origin

fibers seeded with bovine aortic endothelial cells in a biomatrix. The cells can be seen migrating off the polymer into the matrix in a branch-like orientation. Figure 14 is a photograph of bovine aortic endothelial cells attached to polymer fibers after one month in culture. These cells have been shown to reform structure. Their ability to do so depends upon the environment in which they are placed and the degree of alteration they have undergone. In addition to the the bile duct cells which formed tubules in vitro as shown in Figure 6, the aortic endothelial cells attached to polymer fibers formed branching tubule structures after one month in culture. As the polymer fibers resorbed, the cells maintained their orientation, indicating that they secreted their own matrix to maintain their geometric configuration.

Figure 15 is a phase contrast photomicrogrph showing polymer fibers coated with mouse fetal fibroblasts. The fibroblasts can be seen streaming off the polymer fibers in a straight line onto the culture dish. This indicates that cell-cell orientation cues have been maintained as they migrate off the polymer fiber.

Figure 16 is a phase contrast photomicrograph of polymer fibers coated with mouse fetal fibroblasts. These fetal fibroblasts have migrated off of the polymer through media and have attached at the bottom of the tissue culture plate. This shows that a living tissue bridge has been created between the polymer fiber and the tissue culture bottom by fetal fibroblasts, indicating their spatial organization.

These studies demonstrate that cells of liver, intestine, and pancreas will successfully attach and remain viable on polymers in cell culture and that

hour. A combined coating using gelatin and gum arabic was tested. Collagen coated polymers were prepared by covering the polymer with a Type IV collagen and lyophilizing this polymer-collagen material overnight. Some collagen coated samples were immersed in phosphate buffer for one hour. All samples were examined by SEM to determine uniformity of coating. All samples were sterilized using UV exposure under the sterile hood for 8-12 hours. Cells were then added for cell attachment studies.

Cell Attachment Studies.

Cell polymer samples were examined by phase contrast microscopy and SEM using the following sample preparation technique. Samples were fixed by immersion in 50:50 2% gluteraldehyde:phosphate buffer for 1 hour and then rinsed x 3 for 20 minutes with phosphate buffer. They were then dehydrated in progressively increasing concentrations of ethanol solutions (70%, 80%, 90%, 95%) for 20 minutes each, immersed in absolute alcohol overnight, dried by critical point drying with liquid CO_2 and coated with gold.

Isolation and purification of Pancreatic Islet Cells.

Young adult mice were anesthetized and underwent a midline abdominal incision using sterile technique. The common bile duct was isolated and cannulated with a 30 gauge needle. 2.5 cc. of Type IV collagenase was slowly infused through the common bile duct with a clamp on the duodenum so that there would be retrograde flow into the pancreatic duct. The pancreas was then removed and digested with collagenase for 45 minutes at 37°C. The pancreas was then washed with cold Hank's solution and pancreatic tissue passed through a nylon mesh filter. The islets

attachment of pancreatic cells (islets and fibroblasts)on vicryl™ after two weeks in culture. Maximum attachment occurs with polymer coated with crosslinked or uncrosslinked 11% or 1.5% gelatin and collagen. Very little attachment of these cells to polyorthoester and polyanhydride samples was observed. Table V is the attachment of islet cells after two weeks in culture, with maximum attachment again occurring with polymer coated with collagen.

TABLE II

3T3 CELLS ON POLYORTHOESTER

Polymer	Attachment after 2 days	Attachment after 5 days
Control (untreated, with no cells)	Some degradation	Considerable degradation
Untreated	1	1
Agar (5%)	1	1
Agarose (6.7%)	1	1
Gelatin (11%) crosslinked	2	4+
Gelatin (11%) gum 11% crosslinked	4	2
Gum arabic (11%)	1	1
pH 4, 2 days	1	0
pH 4, 5 days	2	1
pH 4, 7 days	1	1
pH 7, 2 days	3	2
pH 7, 5 days	4	3
pH 7, 7 days	2	1
pH 10, 2 days	4+	4+
pH 10, 5 days	0	0
pH 10, 7 days	4	3

Scale

0 No viable cells
1 Minimal cell attachment
2 Moderate cell attachment
3 Good cell attachment
4 Better cell attachment
5 Excellent cell attachment

TABLE IV

PANCREATIC CELLS ON VICRYL® AFTER 2 WEEKS IN CULTURE (MIXTURE OF ISLETS AND FIBROBLASTS)

Polymer	Attachment
Control (untreated, no cells)	Little, if any degradation
Untreated	0
Agar (2%)	1
Agarose (2%)	1
Gelatin (11%) crosslinked	4
Gelatin (11%)	4+
Gelatin (1.5%) crosslinked	2
Gelatin (1.5%)	4+
Gum arabic (1.5%)	1
Gelatin (1.5%)/Gum arabic (1.5%) crosslinked	1
Gelatin (1.5%)/Gum arabic (1.5%)	2
Collagen	4++
Collagen - phosphate buffer	3
pH 4, 2 days	0
pH 4, 4 days	0
pH 7, 2 days	1
pH 7, 4 days	2
pH 10, 2 days	1

Scale

0 No viable cells
1 Minimal cell attachment
2 Moderate cell attachment
3 Good cell attachment
4 Better cell attachment
5 Excellent cell attachment

The method of the present invention is highly versatile and useful both in vivo and in vitro. For example, cells on polymer fibers embedded in Matrigel can be used to create three-dimensional organ structures in vitro. For in vivo applications, the polymer structure is tailored to fit the cells so that the desired function and structure is obtained after implantation, and so that cell growth, proliferation and function can be achieved initially in cell culture. The criteria for successful growth and implantation is when the transplant demonstrates functional equivalency to the organ which it is replacing or supplementing. For example, a functional kidney would not necessarily have to manufacture renin as long as it functions as an effective dialysis apparatus, removing concentrated low molecular weight materials from the bloodstream. A functional liver may only need to produce protein such as coagulation factors and excrete bile. For this purpose the liver transplant could be implanted in the omentum, the fatty, highly vasculated membrane adjacent to the small intestine. A functional intestine should be able to absorb sufficient nutrients to sustain life. This could be in the form of caloric solutions rather than normal "foodstuffs".

"Secretory" organs in addition to a liver or a pancreas can be made by applying the same method of selecting secretory cells, constructing a matrix, culturing the cell on the matrix, and implanting the cell-matrix structure into an area which promotes vasculation of the cell-matrix structure.

As demonstrated in Figure 19, "organs" other than secretory organs can be made using the method of the present invention. Nerves may be constructed

vascularization. Unless the cells are more or less equally exposed to the media, with as shallow of a concentration gradient as possible, this will not occur. As the cells multiply, the passage of nutrients, wastes, and gases to and from the cells becomes limited and the cells farthest from the media die. Since the artificial skin implants were immediately placed on the underlying tissue so that capillary growth into the matrix begins prior to any significant increase in cell density, this has not previously been a consideration.

The concept of Chimeric Neomorphogenesis hinges upon the ability of cells to be nourished by diffusion until vascular ingrowth of the growing cell mass occurs. It was hypothesized that solid implants of a cell-matrix configuration using collagen or gelatin seeded with cells are limited in size by the physical constraints of diffusion. Others are presently using complex natural matrices seeded with cells to produce "organ equivalents". One is a collagen gel that appears to be a hydrated solution of Type I collagen. The following experiment tests the ability of this hydrated collagen to allow diffusion of nutrients to a cell population.

Bovine capillary endothelial cells were plated in gelatin coated 24 well tissue culture dishes and allowed to attach overnight. The initial cell number was 1 x 10^5 cells. The following day the cells were overlayed with different volumes of collagen Type I at a final solution of 0.32%. A standard volume of media was placed over the collagen so that the distance of nutrient source varied to the cells. The media was optimized for growth of bovine capillary endothelium. Dulbecco's minimal essential media, 10% calf serum,

constraints. One would expect that an implant of less than 1 cm^3 would result in cell viability at the periphery of the implant to a depth of 3-5 mm. However, the cells in the center of the implant would not remain viable because of limitation of nutrition, diffusion, as well as gas exchange. One can envision large flat gels with very small thicknesses of 5-10 mm would allow larger implants to occur. However, this two dimensional solution may have geometric constraints for implantation. It is also clear that by increasing cell density, diffusion would be more limited, and, therefore, the distances would be commensurately smaller.

Although this invention has been described with reference to specific embodiments, variations and modifications of the method and means for constructing artificial organs by culturing cells on matrices having maximized surface area and exposure to the surrounding nutrient-containing environment will be apparent to those skilled in the art. Such modifications and variations are intended to come within the scope of the appended claims.

host at the location where said cell-matrix structure is to be implanted.

6. The method of claim 4 further comprising implanting additional cell-matrix structures having different cell populations in conjunction with the first cell-matrix structure.

7. The method of claim 3 further comprising removing lymphocytes from the cell population prior to seeding.

8. The method of claim 3 further comprising modifying the cells to alter the antigen expression on the cell surface.

9. The method of claim 4 further comprising selecting cells of a tissue type compatible with the host's cells.

10. The method of claim 1 further comprising providing compounds selected from the group consisting of nutrients, cofactors, growth factors, compounds stimulating angiogenesis, immunomodulators, inhibitors of inflammation, regression factors, factors stimulating differentiation and dedifferentiation, biologically active molecules stimulating lymphatic network ingrowth, factors enhancing nerve growth and drugs.

11. The method of claim 1 further comprising selecting the biocompatible material for the group consisting of polyorthoesters, polyanhydrides, polyglycolic acid, basement membrane components, agar, agarose, gelatin, gum arabic, collagen types I, II, III, IV, and V, fibronectin, laminin, glycosaminoglycans, and complex mixtures thereof.

12. The method of claim 1 wherein the biocompatible material is selected from the group of

18. The matrix of claim 16 further comprising an overlayer enhancing cell attachment.

19. The matrix of claim 16 wherein said second material is selected from the group of materials which enhance adhesion of cells to the surface of said matrix consisting of agar, agarose, gelatin, gum arabic, basement membrane material, collagens types I, II, III, IV, and V, fibronectin, laminin, glycosaminoglycans, and complex mixtures thereof.

20. The matrix of claim 16 wherein the matrix is disc shaped and has specifically contoured depressions for cell attachment.

21. The matrix of claim 16 wherein said matrix is a fibrous structure.

22. The matrix of claim 21 wherein said fibrous structure includes hollow fibers.

23. The matrix of claim 21 wherein said fibrous structure includes solid fibers.

24. The matrix of claim 16 wherein said matrix is biodegradable.

25. The matrix of claim 16 wherein said matrix is configured as spicules.

26. The matrix of claim 16 further comprising compounds selected from the group consisting of nutrients, growth factors, cofactors, compounds stimulating angiogenesis, immunomodulators, inhibitors of inflammation, regression factors, factors stimulating differentiation and de-differentiation, biologically active molecules stimulating lymphatic network ingrowth, factors enhancing nerve growth, drugs and combinations thereof.

27. The matrix of claim 16 wherein said matrix is configured to provide separate areas of attachment for cells of different origin.

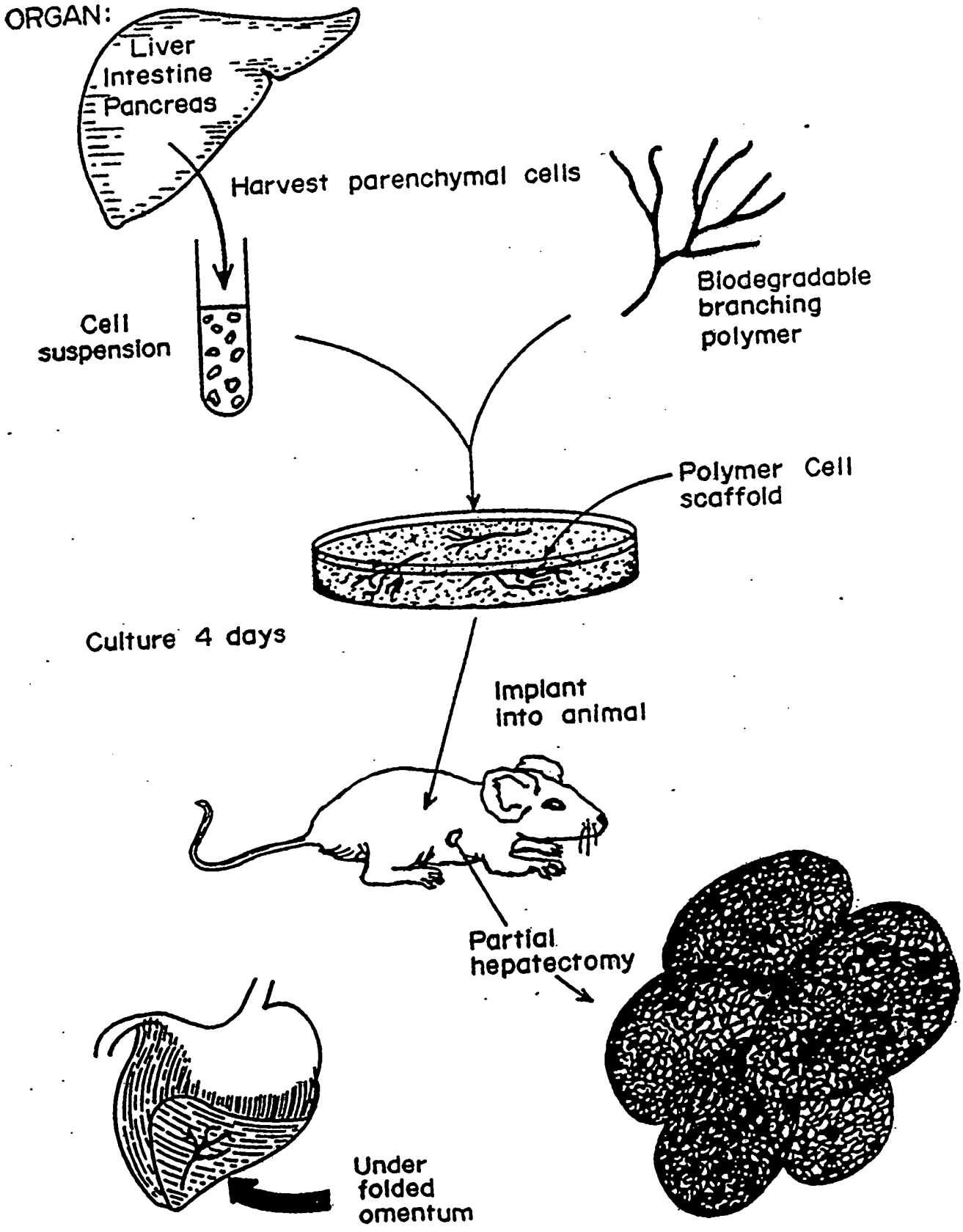

ORGAN:
Liver
Intestine
Pancreas
Harvest parenchymal cells
Biodegradable branching polymer
Cell suspension
Polymer Cell scaffold
Culture 4 days
Implant into animal
Partial hepatectomy
Under folded omentum

FIG 1

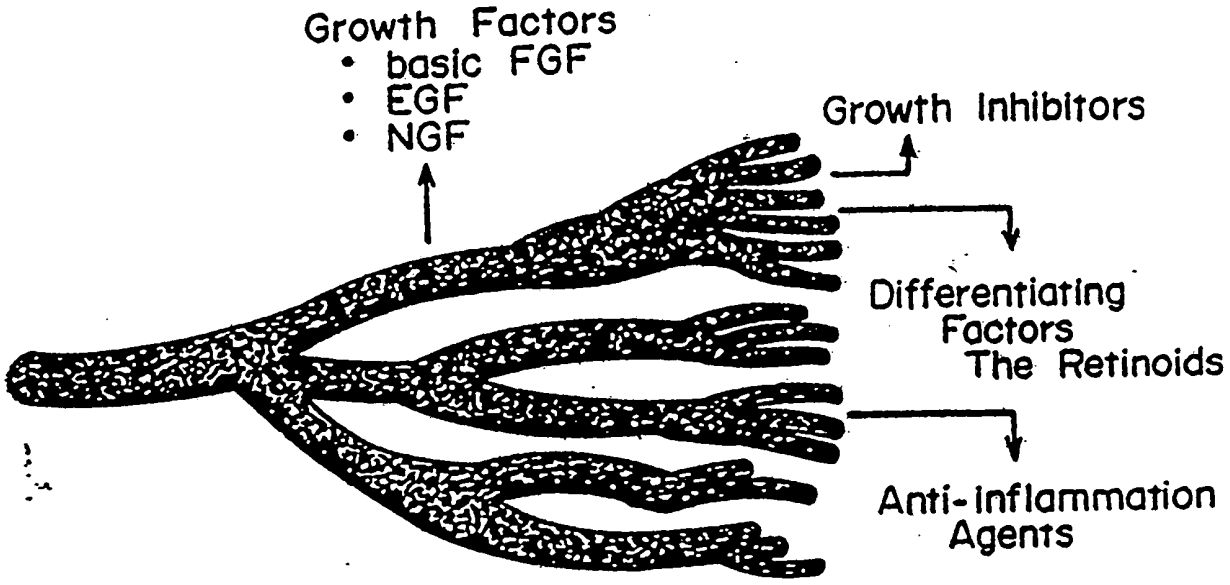

Growth Factors
• basic FGF
• EGF
• NGF
Growth Inhibitors
Differentiating Factors The Retinoids
Anti-Inflammation Agents

FIG.3

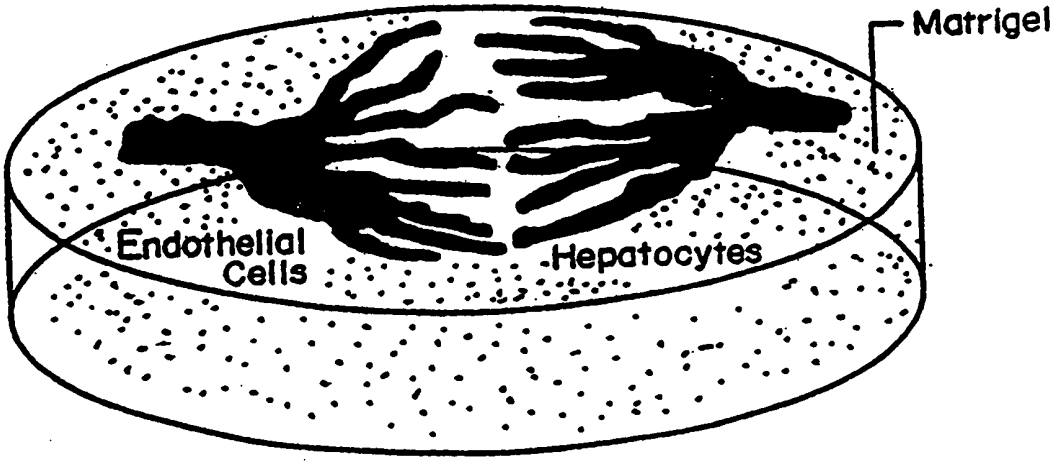

Matrigel
Endothelial Cells
Hepatocytes

FIG.4

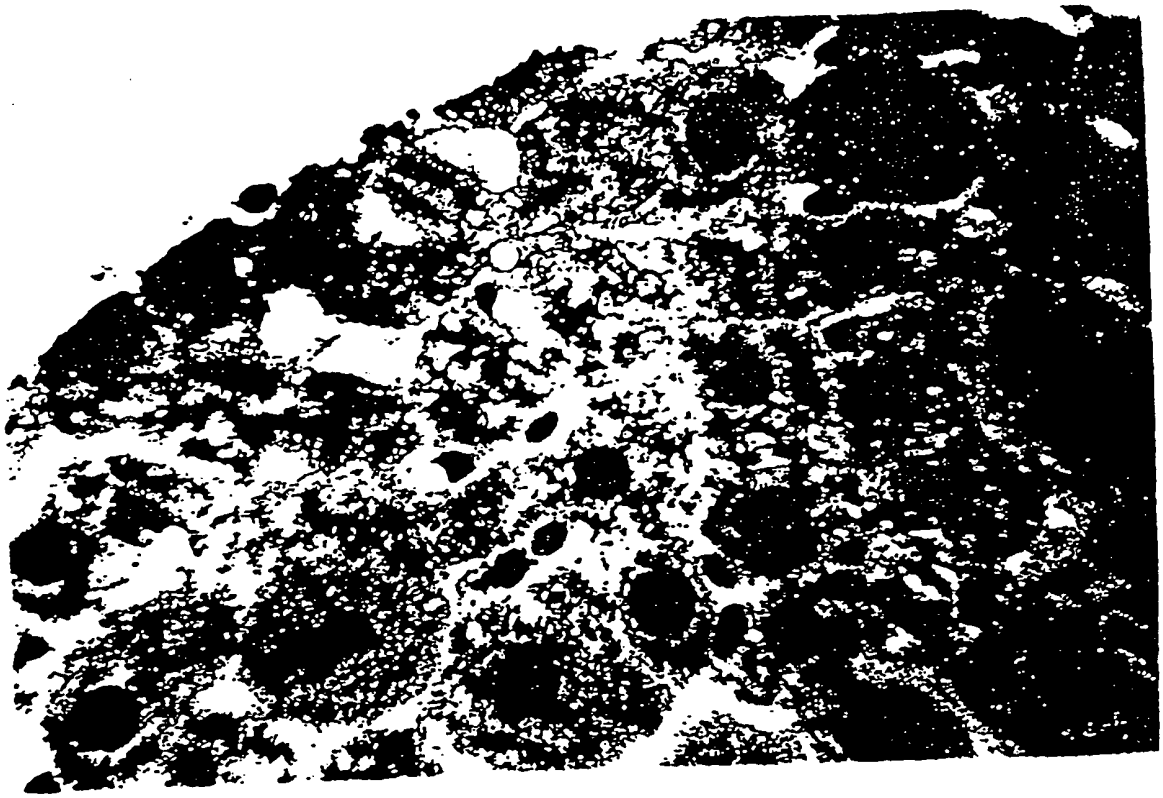


FIG. 7

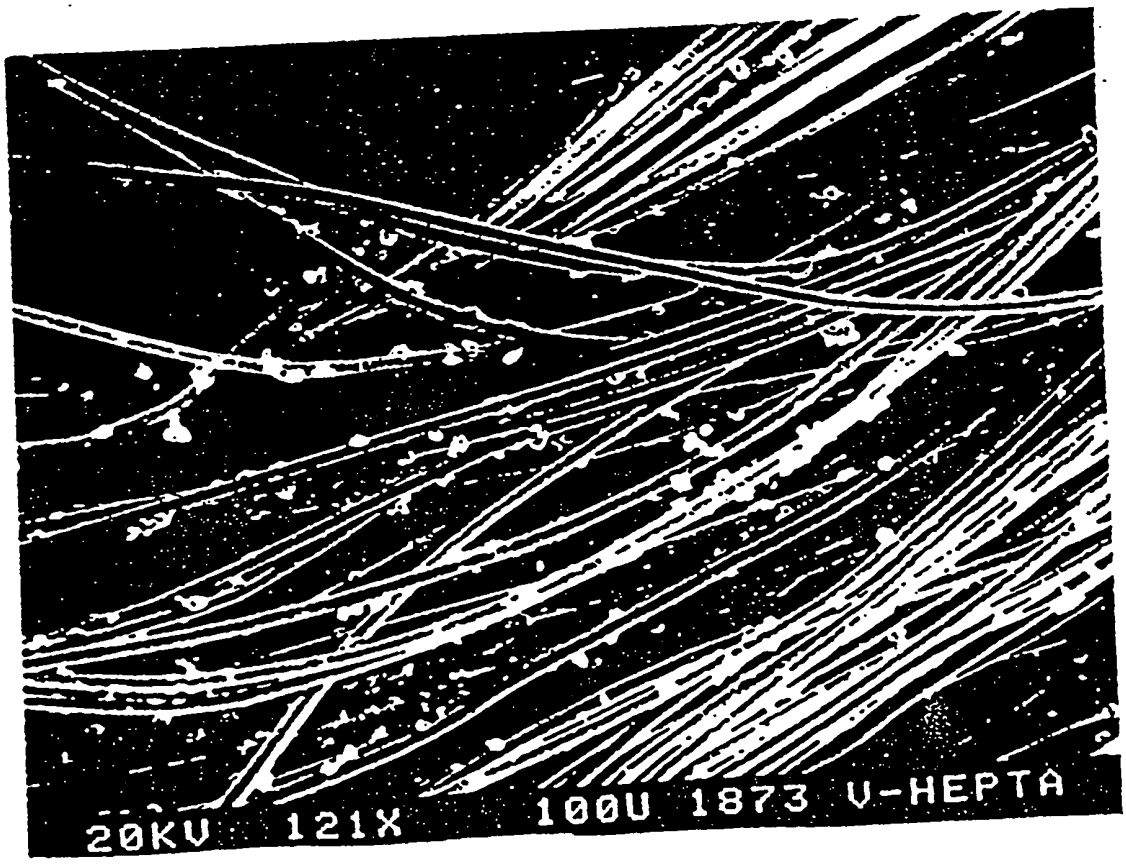


FIG. 8

FIG. 11

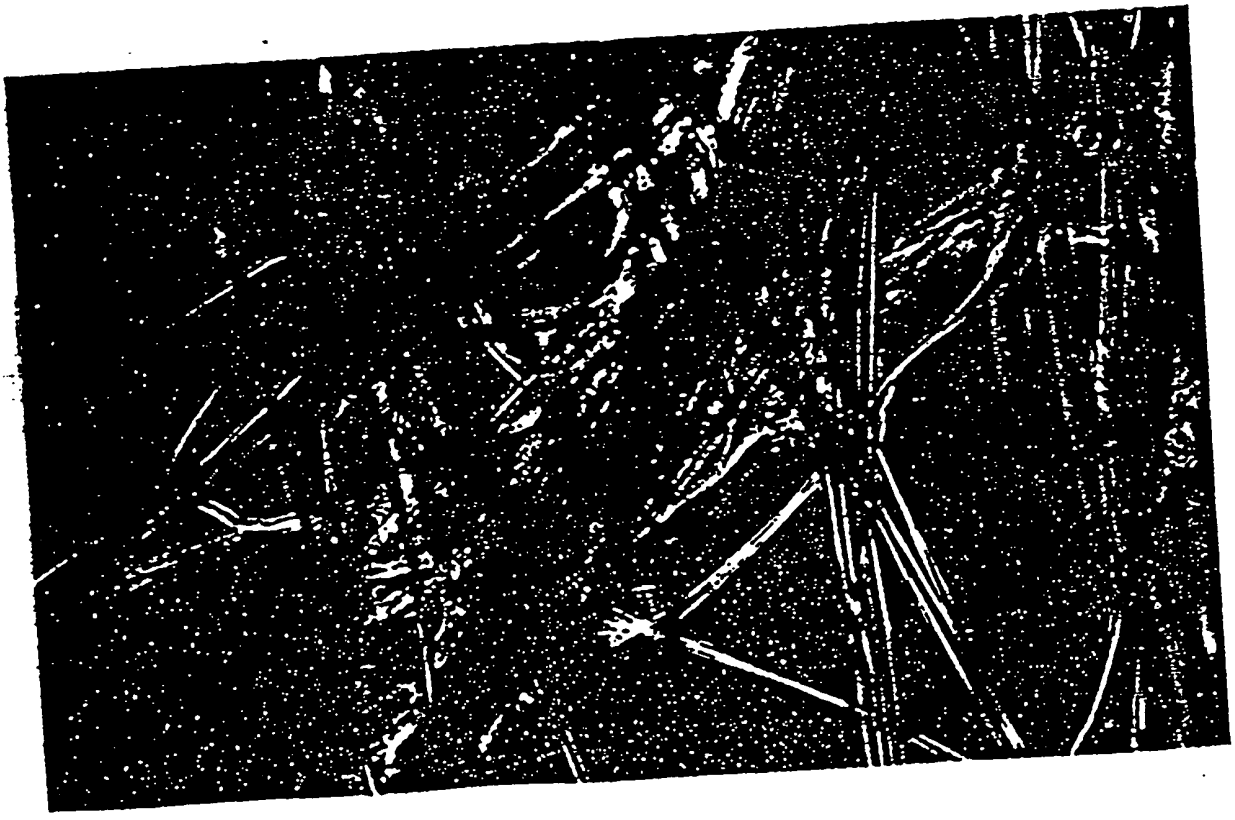


FIG. 12

FIG.15

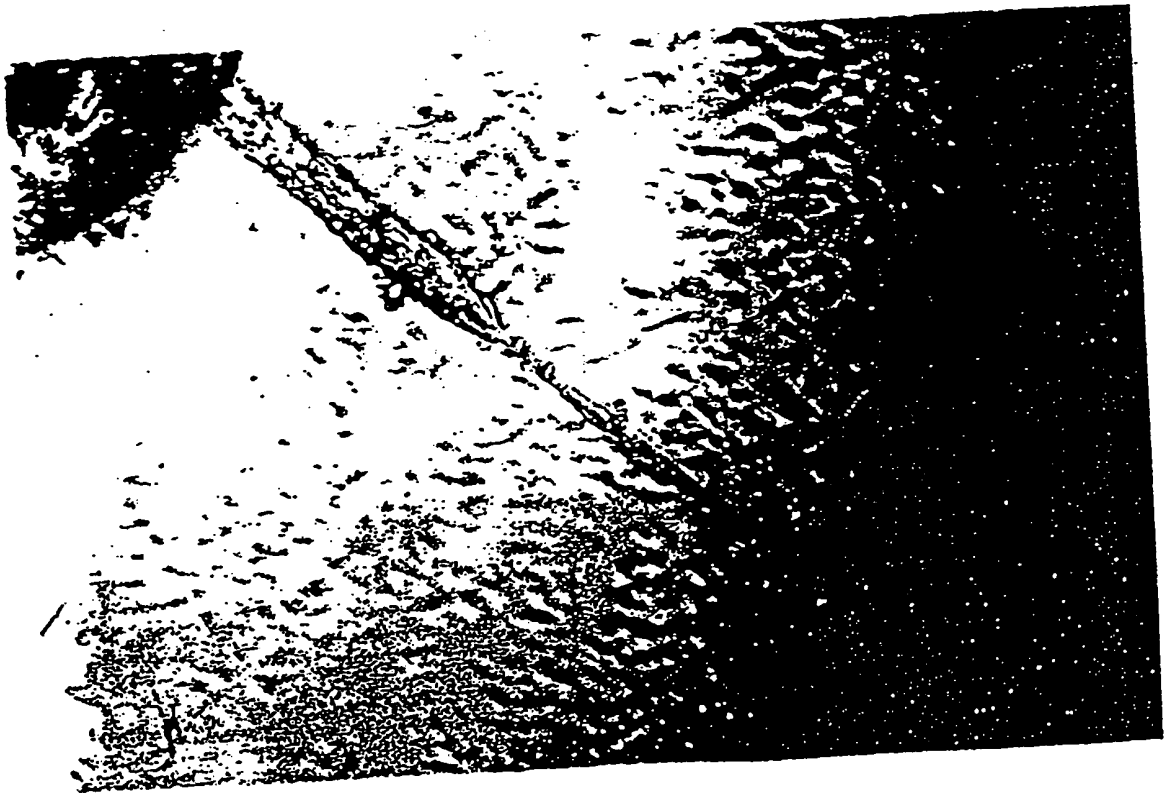


FIG.16

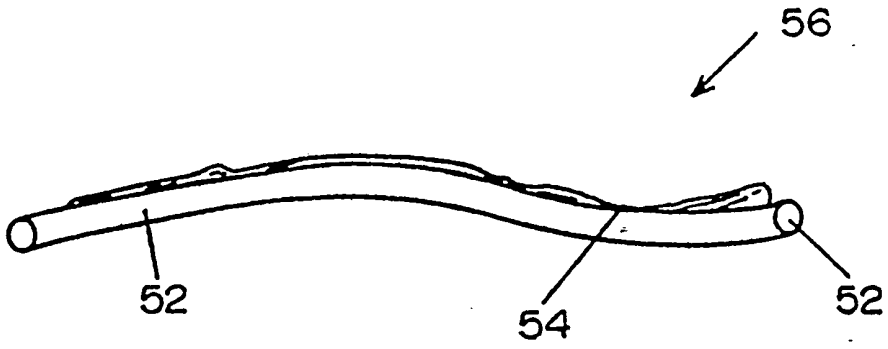


FIG.19

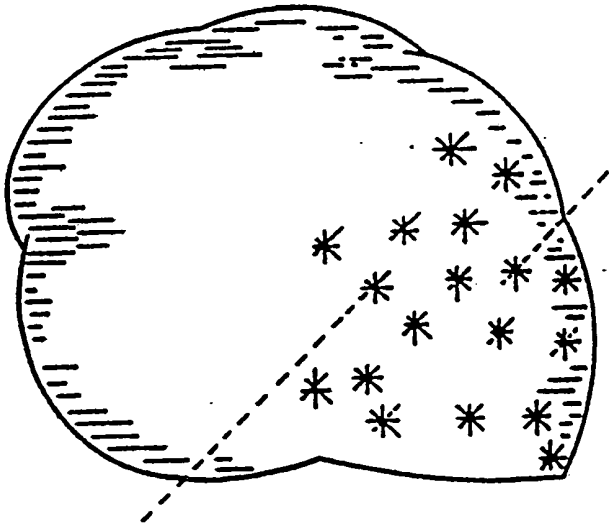
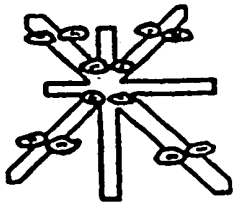


FIG.20a



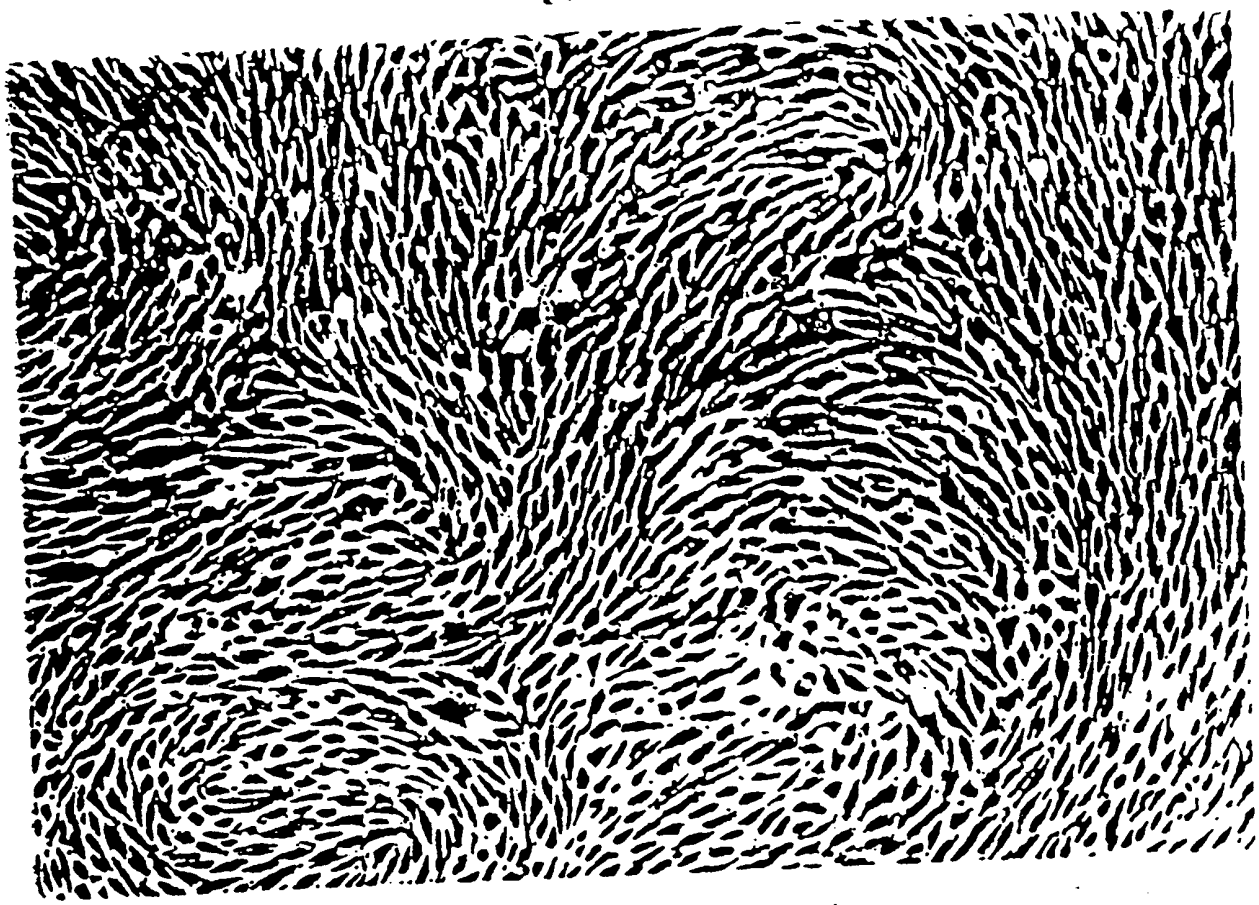
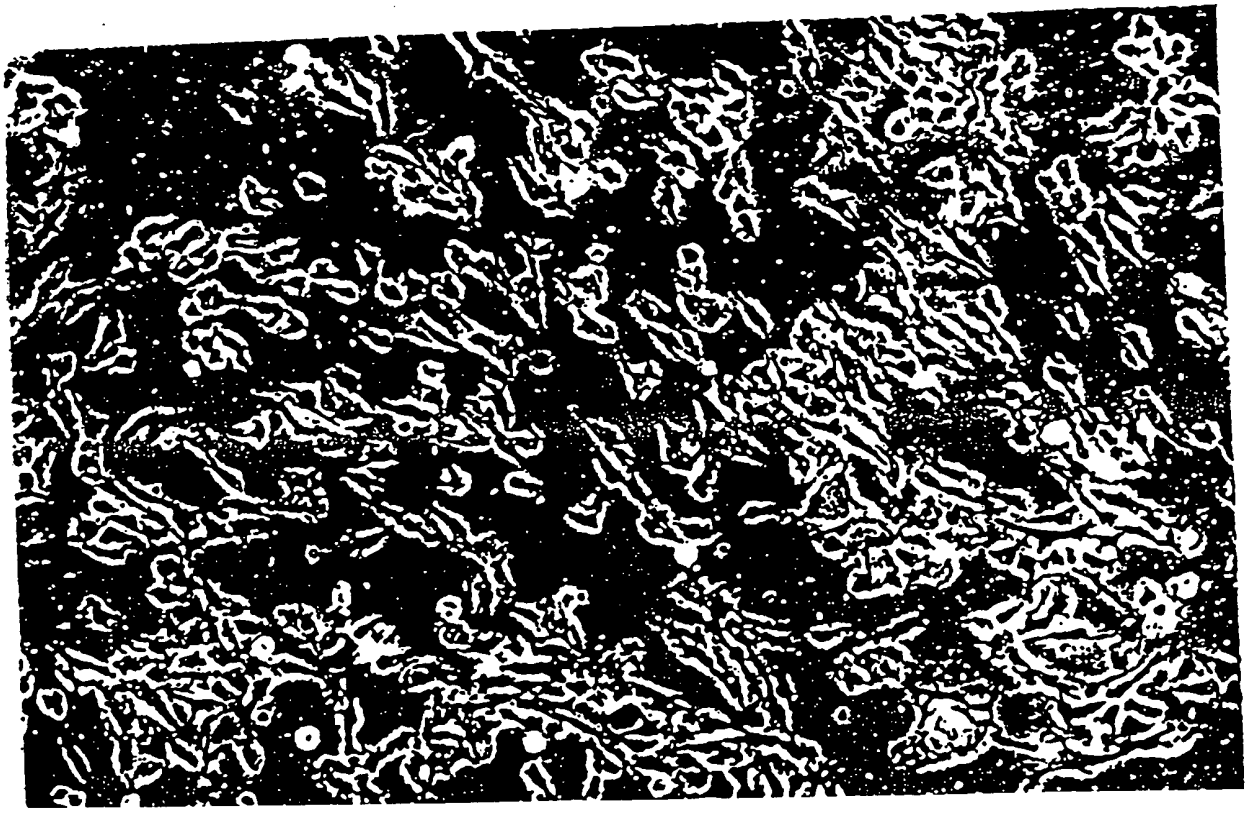


FIG.22a



INTERNATIONAL SEARCH REPORT

International Application No PCT|US87|03091

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4] A61B 19|00; C12N 5|00 U.S. Cl. 435|240.21

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S. Cl.	128|1R; 435|240.21, 240.23, 240.243; 424|422, 423, 486

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	US 4,427,808 STOL, 24 JAN 1984	1-4,9-12,14-17,19,21,24,26 1-29
X Y	US 4,485,097 BELL, 27 NOV 1984	1-4,9-13,15-17,19,21,24,26 1-29
X Y	US 4,458,678 YANNAS, 10 JUL 1984	1-4,8-12,15-19,24,26 1-29
X Y	US 4,060,081 YANNAS, 29 NOV 1977	1,2,11,12,16-19,24 1-29
X Y	US 4,553,272 MEARS, 19 NOV 1985	1,10,16,18,26 1-29
X Y	US 4,559,304 KASAI, 17 DEC 1985	1-3,11,12,16,17,19,24 1-29
Y	US 4,528,265 BECKER, 9 JUL 1985	1-29
Y	US 4,444,887 HOFFMANN, 24 APR 1984	1-29
P,Y	US 4,645,669 REID, 24 FEB 1987	1-29

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]

22 MAR 1988

Date of Mailing of this International Search Report [2]

05 APR 1988

International Searching Authority [1]

ISA|US

Signature of Authorized Officer [20]

Catherine S. Kilby

Catherine S. Kilby